A Radioimmunoassay for Virus Antibody using Binding of 125I-labelled Protein A

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(Accepted 21 November 1978)

SUMMARY

An assay for virus antibodies using protein A from Staphylococcus aureus is described. Type B and type C RNA tumour viruses adsorbed on to polystyrene microtitre plate wells were incubated with antiserum and then with 125I-labelled protein A (I-pA) and bound radioactivity was determined. Technical details such as labelling, antigen concentration, storage of I-pA are reported. The specificity of the reaction was investigated in detail by competition experiments with purified unbound homologous viruses. This assay also proved to be sensitive for demonstration of autogenous immunity to both type B and type C RNA tumour viruses. A study using antisera against purified core and envelope virus proteins of mammary tumour and leukaemia viruses suggested that the reaction mainly involves surface antigens of the intact virions.

INTRODUCTION

Rapid quantification of the extent of humoral immunity to viruses is important as a marker for the virus itself and because it allows an estimation of the immune status of the host during the course of the infection. The level of virus antibodies cannot always be properly detected by standard serological techniques such as neutralization of virus infectivity, precipitation, complement fixation, or cytolysis because the sensitivity of these methods is usually lower than the sensitivity reached by radioimmune assays. However, these techniques have the great advantage of requiring less sophisticated equipment and reagents. On the other hand, the radioimmunoprecipitation test for quantification of virus antibodies (Gerloff et al. 1962; Dalrymple et al. 1972; Wiktor et al. 1972; Ihle et al. 1973; Nowinski & Kaehler, 1974) suffers from the disadvantages that labelling of viruses might be followed by loss or alteration of antigenicity as a consequence of oxidation and radiation damage (Gerloff et al. 1962).

Protein A from Staphylococcus aureus has an affinity for the Fc portion of many immunoglobulin classes of mammalian species (Forsgren & Sjöquist, 1966; Kronvall et al. 1970 a, b; Mackenzie et al. 1978) The use of 125I-labelled protein A for detection of cell bound antibodies has been extensively described (Dorval et al. 1975; Brown et al. 1977; Zelter & Seeger, 1977) and a number of technical details given.

In the present report the reaction of Staphylococcus aureus protein A has been used to measure both natural and immune antibodies bound to purified type B and type C tumour
viruses adsorbed on to polystyrene microtitre plates. Labelling procedures, importance of virus antigen concentration, specificity, reproducibility and sensitivity of the reaction are reported in detail.

METHODS

Viruses. Purified Rauscher-MuLV, Moloney-MuLV, Gross-MuLV, BALB : virus-2 and C3H-MTV were kindly provided by the Office of Program Resources and Logistics, NCI, Bethesda. Ectotropic SJL-MuLV, isolated from high virus expressor mice (Colombatti et al. 1979) and grown on SC-1 cells, was purified from 12 h harvests of culture medium. After clarification at low speed for 10 min the virus was concentrated by pelleting through 5 ml of 25% sucrose in TEN buffer (20 mM-tris-HCl, pH 7.6, 1 mM-EDTA, 100 mM-NaCl) in a Spinco SW 27 rotor at 25,000 rev/min for 1 h. The pellet was layered on a 15 to 60% linear sucrose gradient in TEN and further purified by isopycnic banding at 25,000 rev/min for 12 h. Protein concentration in the virus peak was determined by the Lowry method (Lowry et al. 1951).

Sera. Rabbit antisera against purified disrupted Rauscher-MuLV and purified disrupted C3H-MTV were kindly provided by Dr Ph. Hageman, NKI, Amsterdam. Goat antisera against purified p30 and gp69/71 of Rauscher-MuLV were obtained from the Office of Program Resources and Logistics, NCI, Bethesda. Rabbit antisera against purified p27 and gp52 of C3H-MTV were kindly provided by Dr R. Nusse, NKI, Amsterdam. Mouse antisera against Moloney-MSV were from o2o mice injected 30 days before with Moloney-MSV. SJL sera were from normal SJL mice maintained at the Laboratory of Oncology, Padova.

Protein A iodination. Protein A isolated from a strain of Staphylococcus aureus was purchased from Pharmacia (Uppsala, Sweden) and radioiodinated using the chloramine-T method (Hunter, 1967). Except for chloramine-T and sodium metabisulphite which were diluted in 0.1 M-phosphate, TEN buffer was used throughout.

Briefly, to 1 mCi Na125I (Radiochemical Centre, Amersham, U.K.) were added 15 μl (7.5 μg) of protein A and 10 μl of chloramine-T (2 mg/ml). The reaction mixture was kept for 1 min at room temperature and the reaction stopped by the addition of 5 μl of sodium metabisulphite (20 mg/ml). This concentration of chloramine-T did not seem to impair the functional binding properties of protein A as was reported previously (Dorval et al. 1975) and resulted in protein with high specific activity. Excess cold iodine was then added and the 125I-protein A (I-pA) was separated from free iodine by gel filtration on Sephadex G-25 (fine; Pharmacia, Uppsala, Sweden). Specific activity was determined by TCA precipitation and peak fractions were pooled and diluted in 20 mg/ml of BSA and stored at 4 °C. I-pA was quite stable at this temperature provided it was stored in excess carrier protein. It could be used for more than one month without significant loss of activity. In later experiments BSA was replaced as carrier protein by gelatine because the backgrounds are lower with the latter and because it is also much less expensive. Glass pipettes, tubes and the column were siliconized before use, to prevent sticking of protein A to the glass.

Binding assay for antiviral antibody (I-pA assay). Density gradient purified viruses in TEN buffer (25 μl) were adsorbed to individual wells of polystyrene microtest plates (Falcon 3040) by overnight incubation at 37 °C. Before the addition of antibodies, non-specific sticking to the plastic wells was prevented by incubation for 1 h at 37 °C with 200 μl of 5 mg/ml BSA or gelatine; 25 μl of diluted antiserum (in 1 mg/ml carrier protein) were then added and the plates incubated for 1 h at 37 °C, followed by two washes with washing buffer (0.1 mg/ml carrier protein). Excess I-pA (3 to 5 ng/well) in 25 μl was adsorbed for 30 min at 37 °C or room temperature. Unbound I-pA was removed by three washes with washing buffer.
Virus antibody detected by $^{125}$I-protein A

Radioactivity bound to each well was eluted with $2 \times 200 \mu l$ portions of 2 N-NaOH and counted in a gamma counter. Control wells with no antigen bound represented the background radioactivity which was subtracted to give specific binding.

RESULTS

Typical results of the I-pA assay are shown in Fig. 1 and 2. In this experiment standard sera from rabbits immunized with disrupted viruses were titrated against homologous as well as heterologous viruses. The specificity of the reaction is indicated by the observation that anti-RLV did not bind to C3H-MTV and vice versa; the extent of binding to an unrelated virus was not above the background of antibodies sticking to the plastic. The binding of the anti-FeLV serum to RLV is due to the existence of cross-reacting antigens as already shown by a different approach (Strand & August, 1974). The presence of natural immunity in mice to leukaemia and mammary tumour viruses (Ihle et al. 1973, 1976; Nowinski & Kaehler, 1974) was also detectable with this assay. As an example, the quantitative change in the serum levels of antibodies to SJL ecotropic MuLV in relation to age are shown in Fig. 3. At three months the antiviral activity is low, but by one year it is significantly increased. The binding of protein A was not enhanced when the undiluted serum of young mice was added so that low antibody levels may be determined in this test. The reproducibility of the assay is shown by the results in Table 1.

The extent of binding is affected by the antigen concentration (Fig. 4). In this experiment a standard serum was titrated against decreasing amounts of C3H-MTV; with the drop in concentration of antigen the maximal binding decreased but the antiserum curve was flattened because there was excess antibody for a broader dilution range.

To characterize the binding assay further we analysed the reaction between monospecific sera against purified virus core and envelope proteins of both type B and type C RNA tumour viruses (August et al. 1974; Parks et al. 1974). Plating of virus on polystyrene could have

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Fig. 1. I-pA assay of immune sera to Rauscher-MuLV. Rabbit antiserum against Rauscher-MuLV (○—○), rabbit antiserum against Theilen-FeLV (○—○) and rabbit antiserum against C3H-MTV (▲—▲) were incubated with 1 μg of purified virus. Three ng of $^{125}$I-protein A (sp. act. $8 \times 10^4$ ct/min/ng) were used.

Fig. 2. I-pA assay of immune sera to C3H-MTV. Rabbit antiserum against C3H-MTV (▲—▲), rabbit antiserum against Rauscher-MuLV (○—○), and rabbit antiserum against Theilen-FeLV (○—○) were incubated with 0.8 μg of purified virus. Five ng of $^{125}$I-protein A (sp. act. $3.3 \times 10^4$ ct/min/ng) were used.
Table I. Titration of a standard antiserum against RLV using different batches of \(^{125}\text{I}\)-labelled protein A*  

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* Rabbit antiserum was incubated with 1 \(\mu\)g of Rauscher-MuLV; 3 to 5 \(\mu\)g of \(^{125}\text{I}\)-protein A were used.

produced disruption or distortion of its structure and could therefore have exposed internal antigens as well. As can be seen in Fig. 5 there is considerable reaction between a goat antiserum against RLV gp 69/71 and RLV and MLV even at high dilutions, whereas a goat antiserum against RLV p30 only binds to a very limited extent at low dilutions. Similar results were obtained with rabbit antisera against gp52 and p27 on C3H-MTV (Fig. 6). These data indicate that the internal antigens are not exposed by the plating procedures.

We then examined the effect of addition of unadsorbed virus on antiserum binding to adsorbed virus. Typical competition curves are shown in Fig. 7. When a standard BALB/c serum at a constant limiting dilution is assayed on C3H-MTV in the presence of increasing amounts of unadsorbed C3H-MTV, the binding of antibodies is progressively inhibited. In contrast, RLV did not compete in this assay, even at the highest concentrations. The results of comparable experiments using anti-MSV serum on RLV are shown in Fig. 8. An evaluation of immunological cross-reactivity between RLV Gross-MuLV and BALB: virus-2...
Virus antibody detected by $^{125}$I-protein A

Fig. 5. I-pA assay with monospecific antisera to Rauscher-MuLV proteins. Goat antiserum against p30 and gp70 were incubated with 1 µg of Rauscher-MuLV (●—●) and Moloney-MuLV (▲—▲). Three ng of $^{125}$I-protein A (sp. act. 3.3 x 10$^4$ ct/min/ng) were used.

Fig. 6. I-pA assay with monospecific antisera to C3H-MTV proteins. Rabbit antiserum against p27 (●—●) and gp52 (○—○) were incubated with 0.8 µg of purified virus. Three ng of $^{125}$I-protein A (sp. act. 3.3 x 10$^4$ ct/min/ng) were used.

Fig. 7. Competition for antibody binding by unadsorbed virus. A serum from a mammary tumour bearing BALB/c mouse diluted 1 : 20 was incubated for 30 min with various concentrations of C3H-MTV (●—●) or Rauscher-MuLV (○—○). The mixture was then added to 0.8 µg of bound C3H-MTV and the assay carried out as described in Methods. Four ng of $^{125}$I-protein A (sp. act. 8.6 x 10$^4$ ct/min/ng) were used. The BALB/c serum without competing virus bound 15000 ct/min.

Fig. 8. Competition for antibody binding by unadsorbed virus. Serum from an o20 mouse, in which a Moloney-MSV induced tumour had regressed, was diluted 1 : 40 and incubated for 30 min with various concentrations of Rauscher-MuLV (●—●), Gross-MuLV (○—○), BALB: virus-2 (▲—▲), and C3H-MTV (△—△). The mixture was then added to 0.25 µg of Rauscher-MuLV and the assay carried out as already described. Three ng of $^{125}$I-protein A (sp. act. 8.6 x 10$^4$ ct/min/ng) were used. The o20 serum without competing virus bound 13000 ct/min.

Fig. 9. Competition for antibody binding by unadsorbed disrupted virus. A serum from an o20 mouse, in which a Moloney-MSV induced tumour had regressed, diluted 1 : 50, was incubated for 30 min with various concentrations of intact (●—●) or disrupted Rauscher-MuLV (○—○). The mixture was then added to 0.5 µg of Rauscher-MuLV and the assay carried out as already described. Pelleted virus was treated for 15 min at 37 °C with 0.4% final concentration of Triton X-100. This mixture was then diluted ten times and incubated with the appropriate antiserum dilution. A parallel treatment with the same concentration of Triton X-100 did not significantly alter the adsorbed virus as indicated by a lack of increased binding using a goat antiserum against p30. Three ng of $^{125}$I-protein A (sp. act. 8.6 x 10$^4$ ct/min/ng) were used. The o20 serum without competing virus bound 45000 ct/min.
can also be made from this figure; the type-specific response to Moloney antigens corresponds to the antibodies which cannot be absorbed by GLV or BALB:virus-2. However, in the experiment illustrated in Fig. 9 the binding to adsorbed virus was effectively inhibited by disrupted virus, suggesting that the antigenic determinants are recognized individually and not only when they are present as repetitive units on the intact virus particle.

**DISCUSSION**

The fact that the binding reactions we observed are specific is indicated by the lack of cross-reaction with heterologous sera (i.e. rabbit anti-RLV has no effect on C3H-MTV and vice versa), by the complete inhibition of binding to adsorbed virus by purified homologous virus, the partial inhibition by purified viruses of different subgroups, and finally by the lack of competition with unrelated viruses.

The assay was reproducible not only from day to day but the curve of binding (=ct/min at each serum dilution) was comparable from batch to batch of $^{125}$I-labelled protein A, provided that allowance was made for the differences in specific activity. The sensitivity of the I-pA assay relative to other radioimmunological techniques was not investigated but autogenous immunity to both MuLV and MTV, which is hardly detectable by other serological techniques (Ihle et al. 1973, 1976) was clearly shown. Moreover, since there are no limits to using low serum dilutions such as the interference at high serum protein concentration described for radioimmunoprecipitation (Ihle et al. 1973), very low levels of antibodies can be detected. This raises the question of antiserum titre determination in this assay because, if the antigen is in excess, the radioactivity bound is directly proportional to the antibody concentrations and a plateau is never reached. On the other hand, as the antigen concentration decreases (Fig. 4) and antibodies are in excess, a plateau of maximum binding is evident. A low antigen concentration is probably better for comparing strong antisera; in this case a 50% end-point dilution can be calculated and different sera compared by their titre. In order to reveal all possible antibodies present, weak antisera are better compared when antigen is in excess, and the amount in ng or the number of molecules of I-pA bound at each dilution can be used to show differences in antibody levels.

Protein A has been shown to possess varying affinity for immunoglobulins of different species and classes (Kronvall et al. 1970a; Mackenzie et al. 1978). Therefore, the large range in radioactivity bound among antisera obtained in different mammals depends on the species providing the immunoglobulins to be tested and not necessarily on the level of immune response.

The use of microplates allows analysis of many samples with rapid and easy washing and pipetting. When the responses to several viruses are to be analysed simultaneously the assays are not dependent on individual labelling and are therefore unaffected by the loss of antigenicity which might result from oxidation and the more or less rapid decay of each particular virus; in this respect the stability of radio-iodinated protein A is long lasting, with minimal loss of activity. The range of species to which it can be applied obviates the necessity for specific anti-immunoglobulins for detection of bound antibodies.

As was pointed out previously (Kronvall et al. 1970a; Mackenzie et al. 1978) immunoglobulins may vary in their avidity for I-pA, thus giving a misleading picture of the antiviral immunity. However, each individual immunoglobulin class could be investigated by a indirect assay with a second antibody layer.

The identification of all the individual molecular species recognized by poly-specific antisera is not feasible with the present approach because the antibody-antigen complex cannot be further analysed, but it can be argued that they are envelope antigens as suggested by the results with some sera against purified virus proteins.
Virus antibody detected by $^{125}$I-protein A

This work was supported by Contract NO1 CP 33368 within the Virus Cancer Program of the U.S. National Cancer Institute, by the ‘Consiglio Nazionale delle Ricerche’ contracts 77.00263.84 and 78.00355.84 within the ‘Progetto Finalizzato virus’, and the Associazione Italiana per la Promozione delle Ricerche sul Cancro. Part of the work was undertaken during the tenure of an American Cancer Society—Eleanor Roosevelt—International Cancer Fellowship awarded to A. C. by the International Union Against Cancer. We gratefully acknowledge the technical assistance of Mr P. Moerkerk.

REFERENCES


(Received 4 September 1978)